REMARKS

Claims 3, 4 and 6-24 have been canceled as being drawn to non-elected inventions without prejudice to filing one or more division applications directed to the subject matter of these claims.

Claim 1 has been amended to set forth the SEQ ID NOs of each of the peptides. Claim 5 has been amended to set forth the SEQ ID NOs instead of referring to Table 4. New claims 25-28 have been added to be directed to the elected peptide. New claim 29 has been added to be directed to the elected propeptide.

It is submitted that none of the above amendments are new matter and their entry is requested.

In the Office Action mailed 7 October 2003 2002, the Examiner restricted the claims into four Groups. Applicants elect Group I for examination.

In this Office Action, the Examiner also restricted the claimed subject matter to single conotoxin peptide and its propeptide. As a species of the peptide, Applicants provisionally elect the peptide Bt5 having an amino acid sequence set forth in SEQ ID NO:6 (genus) and SEQ ID NO:138 (species). Claims 1, 2 and 25-28 read on peptide Conotoxin-Bt5. In addition, the corresponding propeptide has the amino acid sequence set forth in SEQ ID NO:73. Claims 5 and 29 reads on this propeptide. This election of a single peptide with its propeptide is made with traverse.

As is well known in the art, a particular class of conotoxins share a conserved cysteine framework, disulfide bridging pattern, conserved non-cysteine residues, and conserved molecular target. For example, it is known that α-conotoxins, the contoxins of the present invention, all share the following conserved four cysteine spacing (CC----C-----C), with the first and third cysteines forming a disulfide bridge and the second and fourth cysteines forming a disulfide bridge. Additionally, almost all α-conotoxins contain a conserved proline between the second and third cysteines. These conserved structural elements serve to form a very characteristic three dimensional structure for the α-conotoxins (see the attached Figure 1). Note that the backbones of each α-conotoxin shown in the attached Figure 1 are superimposable. Other than the conserved elements

mentioned above, the sequences of the α-conotoxins are quite divergent. Similarly, it is known that the ω-conotoxins all share the conserved six cysteine spacing (--C----C---CC---C----C--).

Additionally, the gene organization for all conotoxins has been characterized. As shown in Figure 2 attached hereto, each toxin is found at the C-terminal (3') end of the gene. There are two regions upstream of the toxin sequence in the gene. First, is a signal sequence used to target the protein into the appropriate cellular compartment in the venom-producing cells of the cone snails. This is followed by an intervening pro region whose function has not been determined. Analyses of sequences across all known conotoxin families have determined a very unexpected finding. All members of a conotoxin family share a conserved signal sequence that is different from that of even closely related families. For example, there are two families of conotoxins that share the same cysteine framework and disulfide bridging pattern (--C----C---CC---C----C--). They are the ω-conotoxins and the δ-conotoxins. However, ω-conotoxins all inhibit subtypes of Ca^{+2} channels, while δ-conotoxins all inhibit Na^{+} channel subtypes. Even though these two families share the same cysteine framework and disulfide bridging pattern, they have evolved to inhibit different molecular targets. It was found that the signal sequence of the ω-conotoxins differs significantly from that of the δ-conotoxins. Thus, the sequence of the signal sequence is predictive of a shared target in the nervous system.

The Examiner makes the claim that each sequence requires a separate search, in reasoning why the sequences were patentably distinct. Applicants assert that this is only a result of the limitations in programming of the search engines. There are chemical species of a core peptidic genus. Nothing prevents one skilled in the art from writing a program that would search the peptidic chemical genus as presently exists for the more traditional chemical genus. This lack of programming is due only to the way a skilled artisan would think about peptide chemicals (letter abbreviations, etc.).

Finally, the biological effects of α-conotoxins appear to be diverse when delivered into model animals. It has been well established for EVERY α-conotoxin investigated to date, however, that

they all target nicotinic acetylcholine receptors with high affinity and selectivity. Thus, the conserved elements listed previously serve to confer a specific three-dimensional shape and a conserved function (the inhibition of nicotinic acetylcholine receptors). The conserved three dimensional structure of each conotoxin is equivalent to a conserved chemical core found in the chemical genus often searched and patented by the PTO. The divergent sidechains amount to limited R-groups which are readily searched and allowed by the PTO. To make a distinction between a peptidic chemical genus is arbitrary and capricious.

The divergent biological effects observed for each α-conotoxin are due to differences in function and localization for various nicotinic acetylcholine receptors targeted by the α-conotoxins. Thus, the α-conotoxins form a group of highly structurally and functionally related compounds. The same is true for other families of conotoxins that have been characterized (δ-conotoxins target Na^{+} channels, ω-conotoxins target Ca^{+2} channels, etc.) The Examiner's attention is further directed to McIntosh et al. (*Conus* Peptides as Probes for Ion Channels, Methods in Enzymology, Vol. 294, pp. 605-624, 1999), copy attached hereto, for a review of conotoxin families that goes into detail of the conservation within conotoxin families.

The subject application relates to linear γ-carboxyglutamate rich conotoxins, which each have at least three γ-carboxyglutamate residues. Thus, it is submitted that each sequence given in the claims represents a species of the γ-carboxyglutamate rich conotoxin genus,. Since all the species share a common structural motif, Applicants believe that restriction between the various species of this genus is unwarranted.

Furthermore, there are two criteria for a proper requirement for restriction between patentably distinct inventions: 1) The inventions must be independent or distinct as claimed; and 2) There must be a serious burden on the Examiner if restriction is not required. See MPEP § 803. Examiners must provide reasons and/or examples to support conclusions. For purposes of the initial requirement, a serious burden on the Examiner may be *prima facie* shown if the Examiner shows by appropriate explanation either separate classification, separate status in the art, or a different field

of search as defined in MPEP § 808.02. That *prima facie* showing may be rebutted by appropriate showings or evidence by the applicant. Insofar as the criteria for restriction practice relating to Markush-type claims is concerned, the criteria are set forth in MPEP § 803.02. See MPEP § 803. If the members of the Markush group are sufficiently few in number or so closely related that a search and examination of the entire claim can be made without serious burden, the Examiner must examine all claims on the merits, even though they are directed to independent and distinct inventions. In such a case, the Examiner will not require restriction. See MPEP § 803.02.

Applicants agree that the various conopeptides may be distinct from each other. However, as stated in the MPEP, as discussed above, distinctness alone is not enough to require a restriction. There must also be a serious burden upon the examiner. In the absence of such a burden, the examiner must examine all of the claims (or in this case, it is urged that all of the peptide claims should be examined). It is urged that the burden of examining all of the peptide claims of the present application is not a serious one, and that the burden of examining all of the peptide claims is only slightly greater than examining one of the groups of claims.

The examination entails various aspects. First is a decision concerning utility under 35 U.S.C. §101. Although each peptide species being claimed is distinct, they are all related in their structure and biological activity. Consequently, a decision concerning utility will be identical for all of the species, and there is no added burden of examining all of the species as compared to examining only a single species.

The second aspect of examination is whether the provisions of the various paragraphs of 35 U.S.C. § 112 have been met. In general, and in this case, this means reviewing the application and claims for compliance with the provisions of paragraphs 1 and 2 of § 112. As for the enablement aspect as found in paragraph 1 of § 112, all of the peptides are related in their structure and biological activity. Since no basis for distinguishing between the enablement of one species vs. another species has been set forth, it is presumed that all of the listed peptides will be treated equally. Again, this means that only a single decision needs to be made concerning all of the peptides.

Therefore, this aspect of the examination will not be a serious burden if all peptides are examined, vs. only one of the peptides.

Concerning paragraph 2 of § 112, this involves the wording of the claims. The wording of the claims in each group of claims is identical except for the specified peptide. Consequently, any objections to the language of the claims for one Group of claims is equally applicable to the other Groups of claims. Therefore there is no increase in the burden concerning 35 U.S.C. § 112, second paragraph, if all peptide claims are examined.

The third aspect of examination is a review of prior art to determine whether the claims are anticipated or obvious. There are two aspects of such a search. A first aspect is a review of the prior art literature and patents. The literature to be reviewed will be identical for all of the peptides. All of the claimed peptides have similar, though not identical, structures and all are claimed to have the same utility. The Examiner has not stated that a search of the scientific literature will be any different for one peptide than for any other peptide. Consequently, the search of the patent literature will clearly be the same for all of the peptides. Because the search of the scientific literature and patent literature will be identical for all of the peptides, there is no added burden concerning this aspect if all of the peptides are examined. Furthermore, the search will probably entail a computer search based on the peptide sequences in the sequence listing. It is believed that such a search would identify prior art directed to the claimed peptides or peptides having the specified substitutions.

Consequently, it is submitted that the only reason for restriction is that the peptides are distinct from each other. But as explicitly stated in MPEP § 803, the inventions must be distinct and there must be a serious burden on the examiner. MPEP § 803.02 states that if a search and examination of an entire claim can be made without serious burden, the examiner must examine all claims on the merits, even though they are directed to independent and distinct inventions. As urged above, it is asserted that examination of all of the peptides claims will not impose a serious burden.

In addition, it is submitted that the computer search for the mature toxin will also identify and prior art disclosing the propeptide and will identify prior art disclosing derivatives of the mature

toxin. Consequently no additional searching is required to examine the propeptides and derivatives with the corresponding mature toxins, and thus no undue burden exists in this instance.

In view of the above arguments, it is requested that the restriction requirement imposed in the Office Action mailed 7 October 2003 with respect to individual peptides be reconsidered and that all of the peptides be examined together.

Respectfully submitted,

ROTHWELL, FIGG, ERNST & MANBECK, p.c.

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Attachments: Figure 1 showing three dimensional structure of α-conotoxins
Figure 2 showing gene organization for conotoxins
McIntosh et al., Methods in Enzymology 294:605-624, 1999

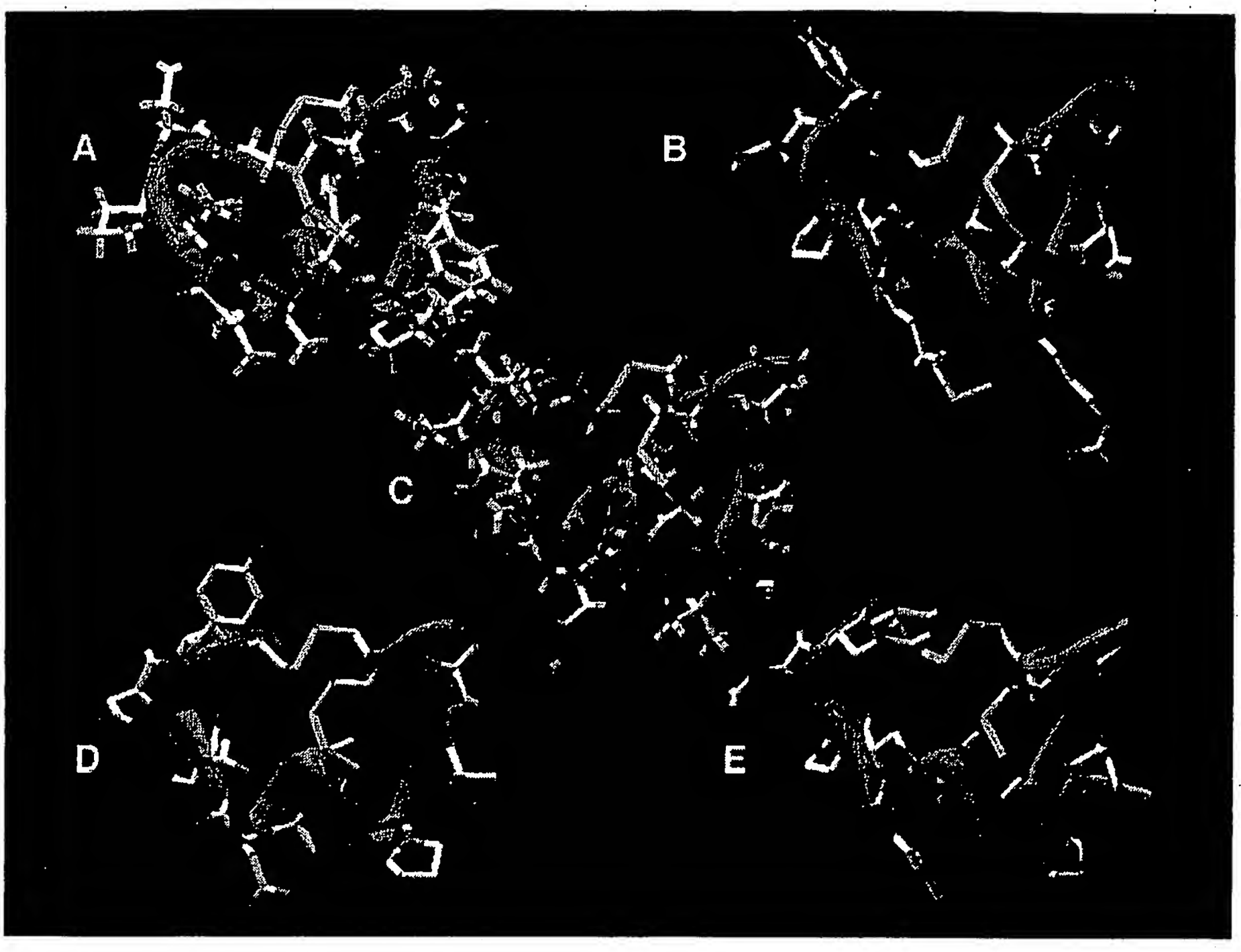

Fig. 1. Backbone structures of several neuronal nAChR-targeted α-conotoxins. A) Structure of α-conotoxin AuIB determined by NMR. B) Structure of α-conotoxin [Tyr^{15}]EpI determined by X-ray crystallography. C) Structure of α-conotoxin MII determined by NMR. D) Structure of α-conotoxin PnIA determined by X-ray crystallography. E) Structure of α-conotoxin PnIB determined by X-ray crystallography.

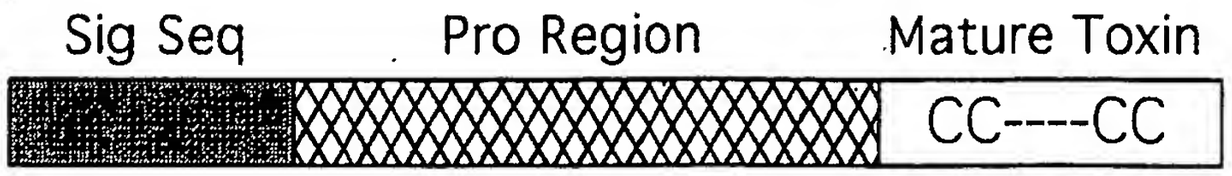

Fig. 2. Gene structure of conotoxins. Every conotoxin isolated to date has a very conserved gene organization. At the N-terminus (5' end of the gene), there is a signal sequence, followed by a pro region in the middle, and the mature toxin at the C-terminus (3' end of the gene). The signal sequence and pro region are removed by processing during maturation to leave only the mature toxin. Each family of conotoxins (each with a conserved target) shares a completely conserved signal sequence. Thus, the signal sequence is completely predictive of the molecular target of the toxin found at the 3' end of the gene regardless of how divergent it may look from other toxins of the same family.

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and the channel hold the promise of resolving this important issue. On a broader front, other toxins offer the possibility to explore different parts of the surface of various channels,[150–152] including sodium channels[153] and potassium channels,[154] and the potential of peptide toxins to reveal details of calcium channel structure remains largely untapped.[150–152,155,156]

Acknowledgments

We thank Dr. Gregory Lipkind, of the University of Chicago Cardiology Molecular Modeling Core, and Chris Bladen for providing figures, and to Dr. Denis McMaster for providing details of the peptide synthesis protocols. Our research is supported by the Medical Research Council of Canada and the National Institutes of Health, USA, P01-HL20592. We are grateful to Dr. Harry Fozzard for numerous discussions, and ongoing support and encouragement. R.J.F. is a Medical Scientist of the Alberta Heritage Foundation for Medical Research and a Medical Research Council Distinguished Scientist.

[150] B. M. Olivera, J. Rivier, C. Clark, C. A. Ramilo, G. P. Corpuz, F. C. Abogadie, E. E. Mena, S. R. Woodward, D. R. Hillyard, and L. J. Cruz, *Science* **249**, 257 (1990).
[151] B. M. Olivera, J. Rivier, J. K. Scott, D. R. Hillyard, and L. J. Cruz, *J. Biol. Chem.* **266(33)**, 22067 (1991).
[152] B. M. Olivera, G. P. Miljanich, J. Ramachandran, and M. E. Adams, *Annu. Rev. Biochem.* **63**, 823 (1994).
[153] J. M. McIntosh, A. Hasson, M. E. Spira, W. R. Gray, W. Li, M. Marsh, D. R. Hillyard, and B. M. Olivera, *J. Biol. Chem.* **270(28)**, 16796 (1995).
[154] K. J. Swartz and R. MacKinnon, *Neuron* **15**, 941 (1995).
[155] I. M. Mintz, *J. Neurosci.* **14(5)**, 2844 (1994).
[156] I. M. Mintz, V. J. Venema, M. E. Adams, and B. P. Bean, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 6628 (1991).
[157] W. A. Catterall, *Curr. Opin. Cell Biol.* **6**, 607 (1994).

[31] *Conus* Peptides as Probes for Ion Channels

By J. MICHAEL MCINTOSH, BALDOMERO M. OLIVERA, and LOURDES J. CRUZ

Introduction

Conus peptides are increasingly used as tools for investigating ion channels. The 500 species of predatory cone snails each produces a complex venom that has a large number of biologically active peptides. The majority of *Conus* peptides characterized to date appear to be targeted to different types of ion channels. It is estimated that the venom of each *Conus* species has between 50 and 200 peptides. Because of the remarkable divergence that occurs when cone snails speciate, the complement of venom peptides

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0076-6879/99 $30.00

in any one *Conus* species is distinct from that of any other. Thus, many thousands of peptides that affect ion channel function are present in *Conus* venoms but only a miniscule fraction of these have been characterized biochemically. An even smaller number have been used as tools in neurobiology. However, there is little doubt that as more of these peptides become available to the neurobiological community, an increasing number will be used as ligands for characterizing ion channel structure and function. Because of their relatively small size, most of these peptides can be chemically synthesized, and thus be made widely available.

Biochemical Overview of *Conus* Peptides

The *Conus* venom peptides can be divided into two general groups: (1) multiply disulfide-bonded peptides from 12 to 50 amino acids in length (most under 30 residues). Generically, these are called conotoxins, and (2) other peptidic venom components that are not disulfide-rich: these either completely lack disulfide bonds or have a single disulfide linkage. The latter are a heterogeneous group of peptides with several distinct families.

In the following sections, we focus first on *Conus* peptides that are targeted to ligand-gated ion channels, followed by peptides that are targeted to voltage-gated ion channels. The last section discusses practical considerations for using *Conus* peptides. It should be noted parenthetically that in much of the literature of the late 1980s and early 1990s, the term *conotoxin* was routinely used to refer to one specific molecule out of the many tens of thousands of *Conus* peptides—this was ω-conotoxin GVIA, the first natural toxin known to inhibit voltage-gated calcium channels. Given the very large number of *Conus* peptides, it is no longer appropriate to use the term *conotoxin* for this one peptide. In this review, *conotoxin* will be used generically for all multiply disulfide-bonded *Conus* peptides.

For neurobiologists, the major interest in *Conus* peptides is that they are highly subtype-specific ligands. For several ion channel targets, *Conus* peptides are the most specific ligands known. For example, among ligands that target voltage-gated sodium channels, μ-conotoxin GIIIA has unprecedented specificity for the skeletal muscle subtype. This isoform is among the set of sodium channels that are tetrodotoxin and saxitoxin sensitive. However, μ-conotoxin GIIIA is much more specific than either of the guanidinium toxins; it has a preference for the skeletal muscle isoform by at least three orders-of-magnitude over other tetrodotoxin-sensitive subtypes.[1,2] This high subtype selectivity is proving to be a general feature of

[1] L. J. Cruz, W. R. Gray, B. M. Olivera, R. D. Zeikus, L. Kerr, D. Yoshikami, and E. Moczydlowski, *J. Biol. Chem.* **260**, 9280 (1985).

[2] T. Gonoi, Y. Ohizumi, H. Nakamura, J. Kobayashi, and W. A. Catterall, *J. Neurosci.* **7**, 1728 (1987).

TABLE I
CLASSES OF *Conus* PEPTIDES AND THEIR MACROMOLECULAR TARGETS

Peptide class	Characteristic structural features (number of amino acids)	Mode of action
α-Conotoxins	CC—C—C (12–19)	Competitive inhibitor of nicotinic ACh receptor
αA-Conotoxins	CC—C—C—C—C (25–30)	Competitive inhibitor of nicotinic ACh receptor
ψ-Conotoxins	CC—C—C—CC (24)	Noncompetitive inhibitor of nicotinic ACh receptor
Conantokins	γ-carboxylate residues. Cys residues not necessary (17–27)	Noncompetitive inhibitor of NMDA receptor
μ-Conotoxins	CC—C—C—CC (22)	Sodium channel blocker; competes with saxitoxin and tetrodotoxin for site I
μO-Conotoxins	C—C—CC—C—C (31)	Sodium channel blocker; does not compete with saxitoxin for site I binding
μ-Conotoxins	CC—C—C—C—C (17)	Blocks molluscan sodium channels
δ-Conotoxins	C—C—CC—C—C (27–31)	Delays sodium channel inactivation: binds to site VI of the channel
κ-Conotoxins	C—C—CC—C—C (27)	Potassium channel blocker
ω-Conotoxins	C—C—CC—C—C (24–29)	Calcium channel blocker

Conus peptides. As a consequence, with more isoforms of ion channel families being cloned and characterized, and the need for subtype-specific ligands increasing, *Conus* peptides will undoubtedly be increasingly used to discriminate functionally between closely related molecular forms of ion channels. In many ways, having a very highly subtype-specific *Conus* peptide ligand provides a complementary approach to having a gene knockout of one particular ion channel isoform.

An overview of the *Conus* peptides known to affect ion channel function is given in Table I.

Conus Peptides Targeting Ligand-Gated Ion Channels

Four families of *Conus* peptides are known to target ligand-gated ion channels; three of these target nicotinic acetylcholine receptors (nAChRs). These include the α-conotoxins, the αA-conotoxins, and the ψ-conotoxins. The first two families are believed to be competitive antagonists of the nicotinic receptor, while the ψ-conotoxins have recently been shown to be noncompetitive antagonists. To date, peptides in all three families have

been found that target the skeletal muscle subtype of nicotinic receptors. However, all *Conus* peptides characterized so far that preferentially inhibit *neuronal* nicotinic receptors belong to the α-conotoxin family.

The other group of peptides that target ligand-gated ion channels is the conantokins; these are unusual *Conus* peptides that have been shown to antagonize the NMDA (*N*-methyl-D-aspartate) subclass of glutamate receptors.

Preliminary evidence for *Conus* peptides that target other ligand-gated ion channels such as the 5HT3 receptor has been obtained, but a complete biochemical characterization of these peptides is not yet published.[3]

Conus Peptides Targeting Skeletal Muscle Subtype of Nicotinic Acetylcholine Receptors

α-Conotoxins

One group of α-conotoxins is known to target the skeletal muscle subtype of nicotinic receptors (the "α3/5 subfamily"). Characteristically, these have three amino acids between the second and third cysteine residues, and five amino acids between the third and fourth cysteine residues of the peptide. The sequences of all α-conotoxins of this subfamily are shown in Table II. Among these is the very first *Conus* peptide that was biochemically characterized, α-conotoxin GI. Certain members of a second subfamily of α-conotoxins, the "α4/7 subfamily" also target the muscle receptor. One example is α-conotoxin EI.[4]

The α3/5 subfamily of α-conotoxins is the best characterized with respect to high targeting specificity for the muscle receptor. α-Conotoxin MI has been shown to discriminate between the α/δ and the α/γ interface of the mammalian nicotinic acetylcholine receptor by approximately 10^4. When the nicotinic receptor from *Torpedo* is used, α-conotoxin SIA has been shown to discriminate totally between the two ligand-binding sites (in this case targeting to the α/γ interface of the *Torpedo* receptor). α-Conotoxins MI and GI have been shown to be inactive at neuronal nAChRs including $\alpha_2\beta_2$, $\alpha_2\beta_4$, $\alpha_3\beta_2$, $\alpha_3\beta_4$, $\alpha_4\beta_2$, and $\alpha_4\beta_4$ subtypes. Additionally, they do not block α_7 and α_9 homomers in contrast to the long α-neurotoxins from elapiid snakes, such as α-bungarotoxin. Thus, compared to α-bungarotoxin, peptides such as α-contoxin MI appear to be much more highly specific.

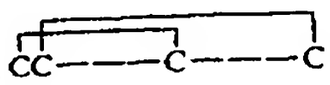
[3] L. J. England, J. Imperial, R. Jacobsen, A. G. Craig, J. Gulyas, J. Rivier, D. Julius, and B. M. Olivera, Seratonin Symposium, San Francisco (1997).

[4] J. S. Martinez, B. M. Olivera, W. R. Gray, A. G. Craig, D. R. Groebe, S. N. Abramson, and J. M. McIntosh, *Biochemistry* **34**, 14519 (1995).

TABLE II

STRUCTURE AND SPECIFICITY OF α-CONOTOXINS

Disulfide bond arrangement:



α-Conotoxin	Source	Primary structure	Site preference	Ref.
Targeted to skeletal muscle nAChR				
	α3/5 Subfamily			
GI	*Conus geographus*	ECCNPACGRHYSC[a]	Mouse: α/δ subunit interface	5–7,42,43
GIA	*Conus geographus*	ECCNPACGRHYSCGK[a]		42
GII[c]	*Conus geographus*	ECCHPACGKHFSC[a]		42
MI	*Conus magus*	GRCCHPACGKNYSC[a]	Mouse: α/δ subunit interface	5–7,43,44
SI	*Conus striatus*	ICCNPACGPKYSC[a]		45
SIA[c]	*Conus striatus*	YCCHPACGKNFDC[a]	*Torpedo*: α/γ subunit interface	46,47
SII[c]	*Conus striatus*	GCCCNPACGPNYGCGTSCS[b]		48
	α4/7 Subfamily			
EI	*Conus ermineus*	RDOCCYHPTCNMSNPQIC[a]	*Torpedo*: α/δ subunit interface	4
Targeted to neuronal nAChRs				
	α4/7 subfamily			
MII	*Conus magus*	GCCSNPVCHLEHSNLC[a]	Rat: $\alpha_3\beta_2$ subunit interface	49
PnIA	*Conus pennaceus*	GCCSLPPCAANNPDYC[a]	*Aplysia*: neuronal nAChR	12
PnIB	*Conus pennaceus*	GCCSLPPCALSNPDYC[a]	*Aplysia*: neuronal nAChR	12
AuIA/B/C	*C. aulicus*	Unpublished	Rat: $\alpha_3\beta_4$ subunit interface	50
	Other			
ImI	*Conus imperialis*	GCCSDPRCAWRC[a]	Rat: α_7 nAChR: *Aplysia*: neuronal nAChR	13,51–53

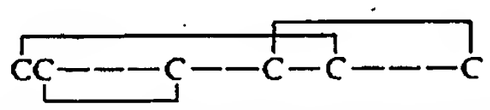
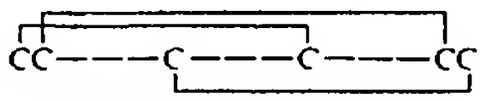
[a] C-terminal α-carboxyl group is amidated.
[b] C-terminal α-carboxyl group is the free acid.
[c] Disulfide bond arrangement has not been determined for GII, SIA, or SII, but very likely is conserved.

It is noteworthy that a small subset of the α3/5 family shows a much greater differential affinity for teleost nicotinic receptors versus mammalian nicotinic receptors. The majority of the peptides in this subfamily (α-conotoxins GI, MI, and SIA) have high affinity for all skeletal muscle nicotinic receptors; in contrast, peptides such as α-conotoxin SI have a dramatically lower affinity for the mammalian skeletal muscle nicotinic receptors.[5]

In contrast to the α3/5 conotoxins which have high affinity for the mammalian α/δ but not the α/γ interface in mammalian muscles, but not

[5] D. R. Groebe, J. M. Dumm, E. S. Levitan, and S. N. Abramson, *Molec. Pharmacol.* **48**, 105 (1995).

TABLE III
STRUCTURE OF αA-CONOTOXINS, ψ-CONOTOXINS, AND CONANTOKINS

Conotoxin	Source	Primary structure[a]	Ref.
Competitive muscle nAChR antagonists Disulfide bond arrangement:			
αA-EIVA	*Conus ermineus*	GCCGPYONAACHOCGCKVGROOYCDROSGG[b]	54
αA-EIVB	*Conus ermineus*	GCCGKYONAACHOCGCTVGROOYCDROSGG[b]	54
αA-PIVA	*Conus purpurascens*	GCCGSYONAACHOCSCKDROSYCGQ[b]	55
Noncompetitive muscle nAChR antagonists Disulfide bond arrangement:			
ψ-PIIIE	*Conus purpurascens*	HOOCCLYGKCRRYOGCSSASCCQR[b]	56
Noncompetitive NMDA receptor antagonists			
Conantokin-G	*Conus geographus*	GEγγLQγNQγLIRγKSN[b]	14,15
Conantokin-T	*Conus tulipa*	GEγγYQKMLγNLRγAEVKKNA[b]	17

[a] γ, γ-Carboxyglutamate; O, *trans*-4-hydroxyproline.
[b] C-terminal α-carboxyl group is amidated.

the α/γ interface in *Torpedo*,[6,7] the α4/7 conotoxin EI shows high affinity for the α/δ interface in both systems and can be used as a selective probe for the α/δ site in *Torpedo*.[4] The structures of several α-conotoxins have been solved both by nuclear magnetic resonance (NMR) techniques and, more recently, by X-ray crystallography.

αA-Conotoxins

Like α-conotoxins of the α3/5 subfamily, αA-conotoxins are believed to be competitive antagonists of skeletal muscle nicotinic receptors (Table III). It has been demonstrated that in contrast to α-conotoxin MI, αA-conotoxin EIVA from the fish-hunting species *Conus ermineus* has almost equal affinity for the two ligand-binding sites of the nicotinic receptor. Indeed, αA-conotoxin EIVA exhibited a higher affinity than any other *Conus* peptide for the α/γ ligand-binding site of the mouse skeletal muscle nicotinic receptor. Thus, α-conotoxins and αA-conotoxins that target the skeletal muscle nicotinic receptor subtype have different specificity for the two ligand-binding sites of mammalian receptors. Clearly, the different

[6] R. M. Hann, O. R. Pagán, and V. A. Eterovic, *Biochemistry* **33**, 14058 (1994).
[7] Y. N. Utkin, F. H. Kobayashi, and V. I. Tsetlin, *Toxicon* **32**, 1153 (1994).

structures reflect different "microsite" interactions[8] even though both groups of peptides are competitive antagonists. The structures of two αA-conotoxins have been solved by NMR.

ψ-Conotoxins

A novel noncompetitive nicotinic receptor antagonist has been described, ψ-conotoxin PIIIE from *Conus purpurascens.* At least two other peptides belonging to this family have been discovered (R. Jacobsen and B. Olivera, unpublished results). ψ-Conotoxin PIIIE has been shown to inhibit the skeletal muscle subtype of nicotinic receptors expressed in oocytes, although it has a significantly higher affinity for the *Torpedo* receptor compared to the homologous mouse receptor. The structure of ψ-conotoxin PIIIE has been determined by multidimensional NMR.

Conus Peptides Targeted to Neuronal Subtypes of Nicotinic Receptors

All *Conus* ligands for neuronal subtypes of nicotinic receptors in mammalian systems belong to the α-conotoxin family. The most specific of such peptides described to date is α-conotoxin MII. This peptide has a very high affinity and target specificity for the $\alpha_3\beta_2$ subtype of neuronal nicotinic receptors. This peptide was used to demonstrate that at least two presynaptic subtypes of neuronal nicotinic receptor are involved in striatal dopamine release, one of which contains an $\alpha_3\beta_2$ interface.[9] Additionally, MII has been used to pharmacologically dissect nicotinically mediated synaptic transmission in chick parasympathetic ciliary ganglion. At this ganglion, MII selectively inhibits the slowly decaying versus rapidly decaying current.[10] A combination of MII and IMI has been used to distinguish subpopulations of nAChRs in frog sympathetic ganglion.[11] The NMR structure of α-conotoxin MII has recently been solved. A variety of data suggest that α-conotoxin MII is a Janus ligand, with two interacting interfaces. One interface is proposed to specifically cause rapid association with the β_2 subunit, and the other to cause functional block and very slow dissociation from the α_3 subunit.

A variety of *Conus* peptides have also been shown to target the α_7 subtype of nicotinic receptors. The first one of these characterized was α-conotoxin IMI from *Conus imperialis* venom. In addition to its specificity

[8] B. M. Olivera, J. Rivier, C. Clark, C. A. Ramilo, G. P. Corpuz, F. C. Abogadic, E. E. Mena, S. R. Woodward, D. R. Hillyard, and L. J. Cruz, *Science* **249**, 257 (1990).
[9] J. M. Kulak, T. A. Nguyen, B. M. Olivera, and J. M. McIntosh, *J. Neurosci.* **17**, 5263 (1997).
[10] E. M. Ullian, J. M. McIntosh, and P. B. Sargent, *J. Neurosci.* **17**, 7210 (1997).
[11] S. F. Tavazoie, M. F. Tavazoie, J. M. McIntosh, B. M. Olivera, and D. Yoshikami, *Br. J. Pharmacol.* **120**, 995 (1996).

for α_7 in mammalian systems, this peptide has been used to discriminate between different types of nicotinic receptors in molluscan systems. Other α-conotoxins have recently been discovered that target the α_7 subtype with significantly higher affinity than α-conotoxin ImI (J. M. McIntosh, unpublished results).

A number of peptides from *Conus aulicus* venom (α-conotoxins AUIA, AuIB, and AuIC), which prefer the $\alpha_3\beta_4$ subtype of neuronal nicotinic receptor, have been characterized. However, the sequences of these peptides have not yet been published.

Some of the α-conotoxins have been shown to act potently at molluscan nAChRs. The first reported peptides were α-conotoxins PnIA and PnIB from *C. pennaceus.*[12] The peptides block the nAChR of cultured *Aplysia* neurons. More recently, α-conotoxin ImI was shown to be a selective antagonist of subpopulations of *Apylsia* nAChRs.[13]

Conus Peptides Targeting NMDA Receptors

The conantokins, which are perhaps the most novel family of *Conus* peptides have been shown to be NMDA receptor antagonists.[14] In contrast to the conotoxins, conantokins are not multiply disulfide-bonded but instead have a very unusual post-translational modification, the γ-carboxylation of glutamate residues to γ-carboxyglutamate (Gla). The discovery of the first member of this family, conantokin-G, established that this unusual post-translational modification could occur outside mammalian systems.[15]

Three conantokins have been characterized so far, conantokin-G from *C. geographus,*[16] conantokin-T from *C. tulipa,*[17] and conantokin-R from *C. radiatus.*[18] These peptides were purified from venom by following an unusual *in vivo* activity in mammals: the ability to induce a sleep-like state in young mice (under 2 weeks of age). Thus, in the earlier papers describing these peptides (before they were found to be NMDA receptor antagonists) they are referred to as "sleeper peptides."

[12] M. Fainzilber, A. Hasson, R. Oren, A. L. Burlingame, D. Gordon, M. E. Spira, and E. Zlotkin, *Biochemistry* **33,** 9523 (1994).
[13] J. Kehoe, M. Spira, and J. M. McIntosh, *Soc. Neurosci.* **22,** 267 (1996).
[14] E. E. Mena, M. F. Gullak, M. J. Pagnozzi, K. E. Richter, J. Rivier, L. J. Cruz, and B. M. Olivera, *Neurosci. Lett.* **118,** 241 (1990).
[15] J. M. McIntosh, B. M. Olivera, L. J. Cruz, and W. R. Gray, *J. Biol. Chem.* **259,** 14343 (1984).
[16] B. M. Olivera, J. M. McIntosh, L. J. Cruz, F. A. Luque, and W. R. Gray, *Biochemistry* **23,** 5087 (1984).
[17] J. A. Haack, J. Rivier, T. N. Parks, E. E. Mena, L. J. Cruz, and B. M. Olivera, *J. Biol. Chem.* **265,** 6025 (1990).
[18] H. S. White, R. T. McCabe, F. Abogadie, J. Torres, J. E. Rivier, I. Paarmann, M. Hollmann, B. M. Olivera, and L. J. Cruz, *J. Neurosci. Abst.* **23,** 2164 (1997).

The conantokins are the only natural peptides known to inhibit NMDA receptors. So far, all natural conantokins tested cause inhibition of a variety of NMDA receptor isoforms, albeit with very different affinities. No other subclass of glutamate receptors that have been examined are inhibited by the conantokin peptides. A report has demonstrated that conantokins have potential as anticonvulsant compounds, exhibiting great potency in an audiogenic seizure mouse model, with a very high protective index compared to commercial anticonvulsant compounds.[18]

Several structural investigations have been carried out on the conantokins using circular dichroism and NMR techniques.[19–21] These studies are in general agreement that conantokins are highly structured peptides, with α-helical structure as well as a distorted 3_{10} helix. For conantokin-G at least, the peptide becomes more structured in the presence of divalent cations. Like the Gla-containing peptides of the blood clotting cascade, conantokin-G binds acidic membranes in the presence of Ca^{2+} ions.[19]

It has recently been shown that the conantokins are initially translated as a large prepropeptide precursor; the mature peptide is found in the C-terminal end in a single copy. In the excised region, which is N terminal to the mature conantokin-encoding C-terminal region, a recognition signal sequence is present that facilitates vitamin K-dependent carboxylation of selected glutamate residues in the mature peptide region.[22] Thus, in contrast to the conotoxins where structure is largely stabilized by the multiple disulfide cross-links, in the conantokin family of peptides the structure is stabilized by the presence of mutiple γ-carboxyglutamate (Gla) residues, appropriately spaced for a helical configuration to be assumed. Sequences in the prepropeptide precursor that do not appear in the mature peptide play an important role in the post-translational conversion of Glu to Gla.

Conus Peptides That Target Voltage-Gated Ion Channels

Overview

The most widely used *Conus* peptides in neurobiology are those that target voltage-gated calcium channels; these all belong to the ω-conotoxin

[19] R. A. Myers, J. River, and B. M. Olivera, *J. Neurosci.* **16**, 958 (1990).
[20] N. Skjaebaek, K. J. Nielsen, R. J. Lewis, P. Alewood, and D. J. Craik, *J. Biol. Chem.* **272**, 2291 (1997).
[21] A. C. Rigby, J. D. Baleja, B. C. Furie, and B. Furie, *Biochemistry* **36**, 6906 (1997).
[22] P. K. Bandyopadhyay, C. J. Colledge, C. S. Walker, L.-M. Zhou, D. R. Hillyard, and B. M. Olivera, *J. Biol. Chem.* submitted (1997).

family (see Table IV). Several different *Conus* peptide families target voltage-gated sodium channels; the first of these discovered were the μ-conotoxins, which are Na^+ channel blockers.[1] The δ-conotoxins are a family of *Conus* peptides that inhibits sodium channel inactivation.[23] Finally, the μO-conotoxins also are sodium channel antagonists,[24] but do not appear to act on the same site as the μ-conotoxins and have a different structural motif (see Table V). The first *Conus* peptide that targets a voltage-gated potassium channel, κ-conotoxin, has been characterized.[25]

Conus Peptides That Target Voltage-Gated Calcium Channels

The literature on the ω-conotoxins that target voltage-gated calcium channels is very extensive, but in this article, only a very brief overview is presented. For a more comprehensive review, the reader is referred to Olivera *et al.*[26] and Dunlap *et al.*[27]

The first ω-conotoxin that was biochemically characterized was ω-conotoxin GVIA from *C. geographus* venom, followed by ω-conotoxin MVIIA from *C. magnus* venom. These were the first natural peptide toxins that inhibited voltage-gated calcium channels. In mammalian systems, these two peptides are very highly subtype-specific, targeting voltage-gated calcium channel complexes that contain an α_{1B} subunit (which correspond to what is known as the "N-type" Ca current).

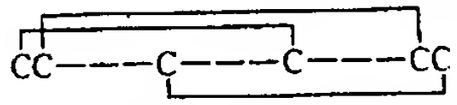
Note that these peptides may have broader selectivity in lower vertebrates (see a discussion in Olivera *et al.*[26]). In the literature, there has been a tendency to assume that any voltage-gated calcium channel that is sensitive to ω-conotoxin GVIA or MVIIA must be α_{1B} containing (i.e., an N-type calcium channel), while any voltage-gated calcium channel resistant to these peptides must be of a different subtype. Although there are no known exceptions so far to this generalization in mammalian systems, there is reason to suspect that the correlation will not hold in lower vertebrates, and almost certainly does *not* apply to invertebrates.

The structures of both ω-conotoxins GVIA and MVIIA have been reported by several laboratories, using multidimensional NMR techniques. Some structure–function studies have been carried out. Both peptides have

[23] K.-J. Shon, A. Hasson, M. E. Spira, L. J. Cruz, W. R. Gray, and B. M. Olivera, *Biochemistry* **33**, 11420 (1994).
[24] M. Fainzilber, O. Kofman, E. Zlotkin, and D. Gordon, *J. Biol. Chem.* **269**, 2574 (1994).
[25] K. Shon, M. Stocker, H. Terlau, W. Stühmer, R. Jacobsen, C. Walker, M. Grilley, M. Watkins, D. R. Hillyard, W. R. Gray, and B. M. Olivera, *J. Biol. Chem.* in press (1997).
[26] B. M. Olivera, G. Miljanich, J. Ramachandran, and M. E. Adams, *Ann. Rev. Biochem.* **63**, 823 (1994).
[27] K. Dunlap, J. I. Luebke, and T. J. Turner, *Trends Neurosci.* **18**, 89 (1995).

TABLE IV

STRUCTURE AND SPECIFICITY OF THE CALCIUM CHANNEL BLOCKERS, ω-CONOTOXINS

ω-Conotoxin	Source	Primary structure	Specificity	Ref.[b]
		Disulfide linkages: 		60,61
GVIA	*Conus geographus*	CKSOGSSCSOTSYNCCRSCNOYTKRCY[a]	N-type calcium channels (α_{1B} subunit)	16
GVIIA	*Conus geographus*	CKSOGTOCSRGMRDCCTSCLLYSNKCRRY[a]		57
MVIIA	*Conus magus*	CKGKGAKCSRLMYDCCTGSCRSGKC[a]	N-type calcium channels (α_{1B} subunit)	58
MVIIB	*Conus magus*	CKGKGASCHRTSYDCCTGSCNRGKC[a]		58
MVIIC	*Conus magus*	CKGKGAPCRKTMYDCCSGSCGRRGKC[a]	P/Q- and N-type calcium channels (α_{1B} and α_{1A})	29
MVIID	*Conus magus*	CQGRGASCRKTMYNCCSGSCNRGRC[a]	P/Q- and N-type calcium channels (α_{1B} and α_{1A})	59
SVIA	*Conus striatus*	CRSSGSPCGVTSICCGRCYRGKCT[a]		48
SVIB	*Conus striatus*	CKLKGQSCRKTSYDCCSGSCGRSGKC[a]	N- and P/Q-type calcium channels (α_{1A} and α_{1B})	48
TxVIIA	*Conus textile*	CKQADEPCDVFSLDCCTGICLGVCMV[c]	Dihydropyridine-sensitive currents in *Aplysia*	30

[a] C-terminal α-carboxyl group is amidated.

[b] See also reviews for primary references.[26,27,62-67]

[c] C-terminal amide is the free acid.

TABLE V

STRUCTURE AND SPECIFICITY OF SODIUM AND POTASSIUM CHANNEL LIGANDS FROM *Conus*

Conotoxin	Source	Primary structure	Specificity	Ref.
		Disulfide linkages: μ-conotoxins (III-family) CC----C----C----CC		23,25,38,72
		μO-, δ- and κ-conotoxins C----C----CC----C----C		
Blockers of voltage-sensitive sodium channels				
μ-GIIIA	*Conus geographus*	RDCCTOOKKCKDRQCKOQRCCA[a]	Skeletal muscle Na channel (μl subtype); binds to site 1	1,68–70
μ-GIIIB	*Conus geographus*	RDCCTPPRKCKDRRCKPMKCCAGR[a]	Same	1,2,68,70
μ-GIIIC	*Conus geographus*	RDCCTPPRKCKDRRCKPMKCCAGR[a]	Same	1
μO-MrVIA	*Conus marmoreus*	ACRKKWEYCIVPIIGFIYCCPGLICGPFVCV[b]	Molluscan neurons (~100 n*M*); type II Na^+ channels and Na^+ channels in cultured rat hippocampal cells; block of rapidly inactivating Ca^{2+} current at higher concentrations (>1 μM)	36–38

μO-MrVIB	*Conus marmoreus*	ACSKKWEYCIVPILGFVYCCPGLICGPFVCV[a]	Same	Same
μ-PnIVA	*Conus pennaceus*	CCKYGWTCLLGCSPCGC[b]	Tetrodotoxin-insensitive molluscan Na^+ channels	39
μ-PnIVB	*Conus pennaceus*	CCKYGWTCWLGCSPCGC[b]	Same	39
Ligands that delay inactivation of voltage-sensitive sodium channels				
δ-GmVIA	*Conus gloriamaris*	VKPCRKEGQLCDPIFQNCCRGWNCVLFCV[b]	Molluscan neurons: shifts voltage-dependent activation curve to more negative potentials and inactivation curve to more positive potentials	23
δ-TxVIA	*Conus textile*	WCKQSGEMCNLLDQNCCDGYCIVLVCT[b]	Molluscan neurons; binding to mammalian Na^+ channels with no apparent physiologic effects and acts to protect against toxic effects of other toxins binding to the same site	24,32,71
δ-PVIA	*Conus purpurascens*	EACYAOGTFCGIKOGLCCSEFCLPGVCFG[b]	Rat brain type II Na^+ channel; rat hippocampal neurons: vertebrate neuromuscular junction	33,34
NgVIA	*Conus nigropunctatus*	SKCFSOGTFCGIKOGLCCSVRCFSLFCISFE[b]	Molluscan and vertebrate Na^+ channels; δ-TxVIA is a partial antagonist of NgVIA	35
Potassium channel blocker				
κ-PVIIA	*Conus purpurascens*	CRIONQKCFQHLDDCCSRKCNRFNKCV[b]	*Shaker* K^+ channel	25,34

[a] C-terminal α-carboxyl group is amidated.
[b] C-terminal α-carboxyl group is the free acid.

been radiolabeled, and used productively in binding experiments, and in autoradiographic studies (for example, see Filloux *et al.*[28]).

In electrophysiologic experiments, ω-conotoxin GVIA is used to inhibit α_{1B}-containing complexes irreversibly, while ω-conotoxin MVIIA is the ligand of choice when a high-affinity but reversible block is desired. Several other homologs of these peptides have been described in the literature (see Table IV).

A second group of ω-conotoxins inhibits both α_{1B}- and α_{1A}-containing calcium channel complexes. These have broader specificity than the α_{1B}-specific ω-conotoxins described above. The most widely used of these peptides is ω-conotoxin MVIIC, which has been used to discriminate between different subclasses of voltage-gated calcium channels. Both ω-conotoxins MVIIC and MVIID clearly inhibit the so-called "P/Q subclasses" of voltage-gated calcium channels, which are widely believed to contain an α_{1A} subunit, although the precise correspondence of P-type and Q-type calcium currents as described by electrophysiologic investigations to α_{1A}-containing calcium channel complexes is still uncertain.

The structure of ω-conotoxin MVIIC has been reported.[29] This peptide has been radiolabeled and used for binding studies. Richard Tsien and coworkers have proposed that ω-conotoxin MVIIC can serve as a key reagent in discriminating between P- and Q-type calcium currents, but this view has not been universally accepted.[27]

Additional ω-conotoxins which inhibit voltage-gated Ca^{2+} channels in invertebrate systems, particularly in mollusks, have been reported.[30] However, although these peptides have been biochemically characterized, their specificity for particular calcium channel subtypes has not yet been established. In certain cases, peptides that were originally isolated as being voltage-gated calcium channel antagonists have proved to be more potent as sodium channel inhibitors.

Conus Peptides That Target Voltage-Gated Sodium Channels

μ-Conotoxins

The μ-conotoxins were the first polypeptide toxins to compete for the same site on Na^+ channels as the well-established guanidinium toxins which

[28] F. Filloux, A. Schapper, S. R. Naisbitt, B. M. Olivera, and J. M. McIntosh, *Develop. Brain Res.* **78**, 131 (1994).

[29] D. R. Hillyard, V. D. Monje, I. M. Mintz, B. P. Bean, L. Nadasdi, J. Ramachandran, G. Miljanich, A. Azimi-Zoonooz, J. M. McIntosh, L. J. Cruz, J. S. Imperial, and B. M. Olivera, *Neuron* **9**, 69 (1992).

[30] M. Fainzilber, J. C. Lodder, R. C. van der Schors, K. W. Li, Z. Yu, A. L. Burlingame, W. P. Geraerts, and K. S. Kits, *Biochemistry* **35**, 8748 (1996).

target sodium channels, tetrodotoxin and saxitoxin. In the nomenclature of Catterall,[31] all of these toxins bind to site I, which is believed to be the outer vestibule of theion channel pore. The μ-conotoxins were originally characterized from *C. geographus* venom, but more recently another μ-conotoxin was isolated and characterized from *C. purpurascens*. As noted earlier, the μ-conotoxins have narrower subtype specificity than the guanidinium toxins. Like the critical guanidinium moiety in saxitoxin and tetrodotoxin, there is believed to be a key arginine in all μ-conotoxins that have been characterized. However, it has been suggested that the guanidinium group of arginine does not in fact interact with the same residues on the voltage-gated ion channel as does the guanidinium group on tetrodotoxin. The structure of several μ-conotoxins, including some analogs, has been described by several groups using NMR techniques.

δ-Conotoxins

The first δ-conotoxin was originally called a "King-Kong peptide" from *C. textile* venom, because it elicited a peculiar symptomatology when injected into lobsters. It was subsequently shown using electrophysiological methods that the peptide delayed inactivation of voltage-gated sodium channels in *Aplysia* ganglion cells.[24,32] Another δ-conotoxin from a snail-hunting *Conus*, δ-conotoxin GmVIA, has also been characterized.[23]

A δ-conotoxin from a fish-hunting cone snail, δ-conotoxin PVIA from *C. purpurascens* venom, has been isolated and chemically synthesized. This peptide has been shown to be important for the very rapid stunning effect of *C. purpurascens* venom on prey.[33,34] This peptide is believed to play a key role in the prey capture strategy of this fish-hunting cone snail.[34] Like the δ-conotoxins from snail-hunting *Conus* venoms, δ-conotoxin PVIA also causes a delay in inactivation.

A conotoxin, NgVIA, that delays inactivation of molluscan and vertebrate sodium channels has been isolated[35] and appears to act on a receptor site distinct from that of δ-TXVIA.

It is notable that although the δ-conotoxins have the same disulfide bonding pattern as the ω-conotoxins, they differ strikingly in the type of amino acids found in the loop regions between disulfide linkages. While ω-conotoxins largely have hydrophilic and positively charged amino acids,

[31] W. A. Catterall, *Physiol. Rev.* **72**, S15 (1992).
[32] S. R. Woodward, L. J. Cruz, B. M. Olivera, and D. R. Hillyard, *EMBO J.* **1**, 1015 (1990).
[33] K. Shon, M. M. Grilley, M. Marsh, D. Yoshikami, A. R. Hall, B. Kurz, W. R. Gray, J. S. Imperial, D. R. Hillyard, and B. M. Olivera, *Biochemistry* **34**, 4913 (1995).
[34] H. Terlau, K. Shon, M. Grilley, M. Stocker, W. Stühmer, and B. M. Olivera, *Nature* **381**, 148 (1996).
[35] M. Fainzilber, J. C. Lodder, K. S. Kits, O. Kofman, I. Vinnitsky, J. Van Rietschoten, E. Zlotkin, and D. Gordon, *J. Biol. Chem.* **270**, 1123 (1995).

in all δ-conotoxins there is a preponderance of hydrophobic residues. It was proposed that the δ-conotoxins bind to a unique site on voltage-gated sodium channels, which has been called site VI. Given the very hydrophobic nature of these peptides, this site may be at least partially in the lipid bilayer.[24]

Because fast inactivation of voltage-gated sodium channels is generally believed to be mediated by a cytoplasmic "ball" region of the ion channel complex, the δ-conotoxins present an intriguing mechanistic puzzle in that they cause an inhibition of fast inactivation from the extracellular side of the membrane.

μO-Conotoxins

Two peptides from the snail-hunting species *C. marmoreus*, μO-conotoxins MrVIA and MrVIB, were shown to block voltage-gated sodium channels.[36,37] They differ from the μ-conotoxins in being more closely related to the δ-conotoxins than to the μ-conotoxins, and also in being the first polypeptide inhibitors that inhibit conductance through Na^+ channels that do not compete for binding with tetrodotoxin/saxitoxin, and clearly target a different site.[38] Furthermore, in contrast to the μ-conotoxins, these peptides act more broadly on different voltage-gated sodium channel subtypes, and a wide variety of different voltage-gated sodium channels are inhibited.

Two conotoxins from *C. pennaceus*, μ-PnIVA and μ-PnIVB, were found by Fainzilber *et al.*[39] to block the tetrodotoxin-insensitive molluscan sodium channels. These peptides are structurally distinct from the originally described μ-conotoxins (e.g., μ-conotoxin GIIIA) and are named with a Roman numeral IV to indicate this difference.

Conus Peptides That Target Voltage-Gated Potassium Channels

So far, only one *Conus* peptide has been shown to inhibit a voltage-gated potassium channel, κ-conotoxin PVIIA from *C. purpurascens* venom. This peptide has a disulfide bonding pattern generally similar to the ω-conotoxins, but instead of inhibiting voltage-gated calcium channels it targets potassium channels. Although the peptide is active both in lower

[36] M. Fainzilber, R. van der Schors, J. C. Lodder, K. W. Li, W. P. Geraerts, and K. S. Kits, *Biochemistry* **34**, 5364 (1995).
[37] J. M. McIntosh, A. Hasson, M. E. Spira, W. Li, M. Marsh, D. R. Hillyard, and B. M. Olivera, *J. Biol. Chem.* **270**, 16796 (1995).
[38] H. Terlau, M. Stocker, K. Shon, J. M. McIntosh, and B. M. Olivera, *J. Neurosci.* (1996).
[39] M. Fainzilber, T. Nakamura, A. Gaathon, J. C. Lodder, K. S. Kits, A. L. Burlingame, and E. Zlotkin, *Biochemistry* **34**, 8649 (1995).

vertebrate systems (where together with δ-conotoxin PVIA, it appears to be responsible for the very fast stunning effect of venom injection on the prey), and shows activity in mammalian systems as well, no vertebrate potassium channel subtype has yet been identified as being targeted by κ-conotoxin PVIIA. However, the well-characterized *Drosophila Shaker* channel is a κ-conotoxin PVIIA target.[25]

There is preliminary evidence for a number of peptides unrelated in structure to κ-conotoxin PVIIA which also inhibit voltage-gated potassium channels. However, the biochemical characterization of these peptides is still in progress, and has not been published. It will be interesting to compare the subtype specificity of these peptides with κ-conotoxin PVIIA. Given the vast diversity of potassium channels, it seems likely that the *Conus* venom system will provide many novel peptides that target potassium channels in the future.

Some Practical Considerations in Handling *Conus* Peptides

Solubility

Conus peptides are soluble in aqueous solutions. In general, a stock concentration of 500 μ*M* may be prepared without difficulty. Some peptides are soluble at higher concentration. Care should be taken, however, to ensure that peptide is actually in solution. Adding buffer to lyophilized peptide often gives the appearance of dissolving the peptide, when, in fact, a suspension has been created. This usually can be detected by holding the mixture up to a light and inspecting for particulates or cloudiness. Examining the solution under a dissecting microscope is often helpful. Certain peptides such as the μO- and δ-conotoxins are much less soluble and require the addition of organic solvents such as dimethyl sulfoxide (DMSO) or acetonitrile to achieve higher micromolar stock concentrations.

Storage

Conus peptides are most stable in lyophilized form. For transport over a few days, they can be safely shipped at room temperature. For longer periods they should be stored at −20° or −80°. Static charge can cause the lyophilized peptide powder to "fly" out of the test tube. If static is encountered, use of an antistatic gun eliminates the problem. Particularly after transport of peptide, it is wise to centrifuge the container to ensure that peptide will not exit the tube on opening. As a side note, peptides lyophilize in a somewhat unpredictable fashion. Small quantities of peptide lyophilized side by side in a rotary evaporator often appear as either a very

visible white powder, or a nearly invisible crystalline substance. The latter can easily be mistaken for "no peptide in the tube" without close inspection.

Peptides solutions can also be stored. For immediate use, solutions are generally kept at room temperature or on ice. For longer storage, solutions are frozen at −20° to −80°. With some peptides we have noted decreased activity after repeated freeze–thaw cycles. High-performance liquid chromatography (HPLC) of these peptide solutions suggests that loss of peptide in solution, rather than peptide breakdown, is occurring. To avoid this, we routinely make aliquots of solutions such that a given aliquot will not need to be thawed more than two or three times prior to consumption.

We often store peptides in HPLC elution buffer consisting of 0.1% trifluoroacetic acid (TFA) and acetonitrile/H_2O. We have found that with long-term storage, however, some peptides (e.g., α-conotoxin EI) undergo degradation, which is consistent with deamination as measured by mass spectrometry. We presume that this is secondary to the acidic pH, and therefore avoid long-term storage under these conditions.

Nonspecific Adsorption

Many *Conus* peptides are hydrophobic in nature and have a tendency to "stick" to glassware and plasticware. At nanomolar concentrations and below, this can lead to significant changes in solution concentration of peptide. To avoid this, we often add 0.1 mg/ml lysozyme or 0.1–1.0 mg/ml bovine serum albumin (BSA) to the solution.

Lyophilization of small quantities of peptide (less than 1 nmol) can lead to significant loss of peptide to container walls. We have found that the addition of carrier protein (e.g., 10–50 μg of lysozyme) to the solution prior to lyophilization largely circumvents this problem. Conodipine-M[40] (a phospholipase A_2 from *C. magus*) is a particularly striking example. The apparent IC_{50} shifts by two orders of magnitude to the right without the utilization of carrier protein.

The use of carrier protein is not always sufficient to prevent nonspecific adsorption, particularly at low peptide concentrations. We have found, for example, that static bath application of α-conotoxins to *Xenopus* oocyte recording chambers leads to an apparent 10-fold decrease in potency compared to preparations where the solution is applied as a continuous flow.[41]

[40] J. M. McIntosh, F. Ghomashchi, M. H. Gelb, D. J. Dooley, S. J. Stoehr, A. B. Giordani, S. R. Naisbitt, and B. M. Olivera, *J. Biol. Chem.* **270**, 3518 (1995).

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[32] Scorpion Toxins as Tools for Studying Potassium Channels

By MARIA L. GARCIA, MARKUS HANNER, HANS-GÜNTHER KNAUS, ROBERT SLAUGHTER, and GREGORY J. KACZOROWSKI

Introduction

Ion channels play a fundamental role in control of cell excitability. Thus, their activity is largely involved in modulation of contractility of muscle cells, and in release of hormones and neurotransmitters from endocrine and neuronal cells. Out of all the families of ion channels, K^+ channels represent the largest and most diverse group of proteins. Gating of these proteins occurs through conformational changes that are controlled by voltage and/or ligand binding. Therefore, K^+ channels can be broadly divided into two groups: voltage-dependent and ligand-activated channels. A number of techniques have become available during the last few years for studying ion channel structure and function. Electrophysiology affords determination of biophysical parameters that are inherent to each individual ion channel. With the use of molecular biology, a large amount of information regarding the structure and existence of subfamilies of K^+ channels has become available due to molecular cloning of cDNAs encoding these

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